Inflammation-induced cholestasis in cancer cachexia

Morgane M. Thibaut[1], Martina Sboarina[1], Martin Roumain[2], Sarah A. Pötgens[1], Audrey M. Neyrinck[1], Florence Destrée[1], Justine Gillard[1,3], Isabelle A. Leclercq[3], Guillaume Dachy[4], Jean-Baptiste Demoulin[4], Anne Tailleux[5], Sophie Lestavel[5], Marialetizia Rastelli[1,6], Amandine Everard[1,6], Patrice D. Cani[1,6], Paolo Porporato[7], Audrey Loumaye[8], Jean-Paul Thissen[8], Giulio G. Muccioli[2], Nathalie M. Delzenne[1], Laure B. Bindels[1#]

 # Correspondence: Laure B. Bindels, [1]Metabolism and Nutrition Research Group, Louvain Drug Research Institute, UCLouvain, Université catholique de Louvain, Brussels, Belgium

Email: laure.bindels@uclouvain.be

SUPPLEMENTARY EXPERIMENTAL PROCEDURE

Mouse experiments

The experiment regarding the administration of a neutralizing IL-6 antibody was performed as previously described [1]. Briefly, this experiment was composed of 4 groups of mice: CT group (sham-injected), C26 group (receiving an injection of C26 cancer cells and treated with vehicle (phosphate-buffered solution)), anti-IL6 group (receiving an injection of C26 cancer cells and treated with 300 μg monoclonal rat anti-murine IL-6 antibody (clone MP5-20F3, BioXCell, NH, USA)) and IgG group (receiving an injection of C26 cancer cells and treated with 300 μg rat IgG1 isotype control (catalogue # BE0088, BioXCell, NH, USA)). Treatments consisted of subcutaneous injections on days 7 and 9 after cancer cells administration.

The pair-feeding experiment was performed as previously described [1]. Briefly, the pair-feeding experiment was composed of 4 groups of mice: CT group (sham-injected and fed *ad libitum*), C26 group (receiving an injection of C26 cancer cells and fed *ad libitum*), PF-CT group (sham-injected and fed the mean amount consumed by the CT mice) and PF-C26 group (sham-injected and fed the mean amount consumed by the C26 mice). Pair-fed mice received daily in 2 equal portions the amount of food consumed by the group they were matched to, with one week delay.

The kinetic experiment was composed of 6 groups of mice: CT-D8 group (sham-injected and euthanized 8 days after injection), C26-D8 group (receiving an injection of C26 cancer cells and euthanized 8 days after injection), CT-D9 group (sham-injected and euthanized 9 days after injection), C26-D9 group (receiving an injection of C26 cancer cells and euthanized 9 days after injection), CT-D10 group (sham-injected and euthanized 10 days after injection), C26-D10 group (receiving an injection of C26 cancer cells and euthanized 10 days after injection).

Western blot analyses

Extraction of nuclear proteins in the liver was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermoscientific, 78835). Protein concentration of portal vein samples or hepatic nuclear extracts was measured using the Lowry method (DC Protein Assay Bio-Rad).

Portal vein proteins (40µg) were separated by 15% SDS-PAGE and transferred to nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) before blocking in Tris-buffered saline–Tween 20 (TBST) containing 5% nonfat dry milk (Bio-Rad) for 1 h at room temperature. Membranes were incubated overnight at 4°C with FGF15 primary antibody (dilution 1/4000 in 1% BSA TBS-T; Cell Signaling Technology, Danvers, MA, USA) (Abcam: ab229630). After membrane washing, horseradish peroxidase–linked secondary antibodies (Goat Anti-Rabbit IgG (Millipore- AP 132); 1/20000 in 1% nonfat milk) were incubated for 1 h at room temperature. Signals were revealed using the SuperSignal West Pico and Femto Chemiluminescent substrates (Thermo Fisher Scientific) and analyzed with the ImageQuant TL instrument and software v.8.1 (GE Healthcare, Waukesha,WI, USA). The loading control was performed using the detection of Blue Coomasie (Coomassie Brilliant Blue G-250, Bio-Rad). Membranes were washed with distilled water and then incubated with Blue Coomasie during 5 min to obtain the required coloration. To stop coloration, they were washed again with distilled water. Signals were analyzed with the ImageQuant TL instrument and software v.8.1.

Nuclear extract proteins (40µg) were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) before blocking in Tris-buffered saline–Tween 20 (TBST) containing 5% nonfat dry milk (Bio-Rad) for 1 h at room temperature. Membranes were incubated overnight at 4°C with NF-kB p65 primary antibody (dilution 1/1000 in 1% BSA TBS-T; Cell

Signaling Technology, NF-kappa B Pathway Sampler Kit 9936T) or TATA primary antibody (dilution 1/1000 in 1% BSA TBS-T; Cell Signaling Technology, NF-kappa B Pathway Sampler Kit 9936T). After membrane washing, horseradish peroxidase–linked secondary antibodies (Goat Anti-Rabbit IgG (Millipore- AP 132); 1/1000 in 1% nonfat milk) were incubated for 1 h at room temperature. Signals were revealed using the SuperSignal West Pico and analyzed with the ImageQuant TL instrument and software v.8.1 (GE Healthcare, Waukesha,WI, USA).

Bile acid quantification

Bile acid quantification was performed mostly as previously described [2]. Briefly, tissue samples (or freeze-dried feces) were homogenized in ice-cold distilled water and proteins were precipitated, in the presence of deuterated internal standards, using acetone. The samples were next centrifuged, the supernatant recovered and evaporated to dryness. The resulting residue was analyzed by HPLC-MS using an LTQ-Orbitrap coupled to an Accela HPLC system (ThermoFisher Scientific). Analyte separation was performed on an Ascentis Express C-18 column (2.7 µm, 4.6 × 100 mm) (Sigma-Aldrich). The separation was achieved using a gradient of H_2O-ACN-formic acid 75:25:0.1 (v/v/v) and ACN-formic acid 100:0.1 (v/v) and the MS analysis was performed in the negative mode with an ESI ionization source. Calibration curves were prepared using the same conditions. Data are expressed as pmol normalized by the amount of tissue. Values below the LOQ (for datasets with less than 25% of such missing values) were imputed using the function *impute.QRILC* in the R package *imputeLCMD*. Total bile acid quantification in the serum of colorectal cancer patients was performed using the Diazyme Total Bile Acids Assay Kit (Diazyme, DZ042A).

Hepatic whole transcriptome

Hepatic RNA samples were sequenced after polyA selection using a 2x150 Paired End (PE) configuration on an Illumina HiSeq 4000 instrument (Genewiz, Germany). RNA sample quality assessment, RNA library preparation, sequencing and raw data analysis were conducted at GENEWIZ, Inc. (South Plainfield, NJ, USA). RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA). Excess DNA was removed by DNase I treatment (Qiagen).

RNA sequencing library preparations used the NEBNext Ultra RNA Library Prep Kit for Illumina following manufacturer's recommendations (NEB, Ipswich, MA, USA). Briefly, mRNA were first enriched with Oligod(T) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First strand and second strand cDNA were subsequently synthesized. cDNA fragments were end-repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment with limited cycle PCR. The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA).

The sequencing libraries were clustered on two lanes of a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq 4000 instrument (or equivalent) according to manufacturer's instructions. The samples were sequenced using a 2x150 Paired End (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS). Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. One mis-match was allowed for index sequence identification. Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software.

Raw fastq files were analyzed *in house* using well-established bioinformatics tools. Briefly, sequences were quality-controlled using FastQC tool (Babraham Bioinformatics, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed by Trimmomatic Version 0.38 [3]. Sequenced reads were then aligned to the annotated reference genome of *Mus musculus* GRCm38.75 (http://labshare.cshl.edu/shares/gingeraslab/www-data/dobin/STAR/STARgenomes/ENSEMBL/mus_musculus/ENSEMBL.mus_musculus.release-75/) using STAR Version 2.6.1d [4]. Count matrices were generated from reads aligned to the reference genome (BAM files) using the function *summarizeOverlaps* from the R package *GenomicAlignments* [5] and then tested for differential expression and normalized using the R package *DESeq2* Version 1.22.2 [6]. A Gene Set Enrichment Analysis using R package *fgsea* Version 3.8 was carried out and pre-ranked genes were analyzed with the *Mus musculus* Molecular Signature Database (MSigDB) [7]. The raw RNA sequences generated and analyzed for this study can be found in the GEO (Gene Expression Omnibus) database (project ID: GSE154219).

Cross-sectional prospective study with cancer patients

The cohort of patients and its characterization was previously reported [8]. This cross-sectional prospective study was performed at the Cliniques universitaires Saint-Luc, Brussels, Belgium. The protocol was approved by the ethics committee of the Université catholique de Louvain (NCT01604642).

Patients with colorectal cancer, confirmed by anatomopathology, were recruited at the diagnosis or at relapse, before any therapeutic intervention, from January 2012 to March 2014. Written consent was given prior to entry into the study. Exclusion criteria were: non-Caucasian subjects, obvious malabsorption, major depression, artificial nutrition, high doses of steroids (>1 mg/kg hydrocortisone equivalent), hyperthyroidism, other causes of malnutrition, major walking handicap, ECOG performance status ≥4 and psychological, familial, social or geographic conditions that would preclude participation in the full protocol. The cachectic status was determined according to the definition proposed by Fearon et al, as an involuntary weight loss > 5% over the past 6 months or weight loss > 2% and body mass index < 20 kg/m² or weight loss > 2% and low muscularity (LM). Overall survival was analyzed since the day of the inclusion visit to 24 months later. Appetite was evaluated by the Simplified Nutritional Appetite Questionnaire (SNAQ) score [8, 9]. The functional status was assessed by a previously validated scale, namely the EORTC QoL questionnaire (QLQ-C30) [10]. Skeletal muscle strength was assessed with a Jamar hand-held dynamometer. Three measures were made on the non-dominant side in a time interval of 30 seconds. The highest value was retained. Muscle strength ratio was calculated as hand grip strength (kg)/hand grip strength (kg) 50th percentile for age and for sex. Skeletal muscle mass (referred here as “lean mass”) were assessed by abdomen CT scan as described by Loumaye and colleagues [8].

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